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Isolation and Characterization of a Mouse Cell Line Containing a Defective Moloney Murine Leukemia Virus Genome

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A culture of mouse cells containing a 1,000-nucleotide deletion mutant of Moloney murine leukemia virus has been isolated. The deletion did not affect the size or function of the 21S mRNA that encodes the *env* gene products. Both the deleted RNA and the 21S mRNA were recovered in polyribosomes. Cells containing the deleted virus made no detectable $Pr180^{gag\text{-}pol}$. $Pr65^{gag}$ synthesis was also absent, but a 45,000-molecular-weight *gag* gene product was found that might be encoded by the deleted genome. Biosynthesis of $Pr80^{env}$ proceeded normally in these cells; the intracellular precursor was cleaved and migrated to the cell surface as gp70. The cells could not be superinfected by homologous Moloney murine leukemia virus presumably because of surface restriction due to the gp70. Although the cells express the Moloney murine leukemia virus gp70 on their surface, they will not make pseudotypes after infection with vesicular stomatitis virus implying that $Pr65^{gag}$ may play a critical role in pseudotype formation. Induction of endogenous virus expression in the cells carrying the deletion mutant generated an N-tropic murine leukemia virus that can fuse XC cells. This may represent a recombinant between the deletion mutant and an endogenous virus.

Although infections of mouse cells by a murine leukemia virus (MuLV) are usually thought to result in a virus-producing infected cell, there are reports of nonproductive cells that produce viral antigens and were therefore infected (42, 47). Such a state is also seen in cultured normal cells from certain mouse strains and reflects expression of endogenous viral genomes; in fact, most mouse cells express some viral antigen but make no virus (6, 32, 38, 51).

A few years ago we noted that a culture of cells infected with Moloney MuLV (M-MuLV) was producing decreasing yields of virus with continuous passage. We have recovered from that culture a nonproducer cell line that expresses viral glycoprotein but little of the other viral proteins. Cells productively infected with M-MuLV make gene products from the three known genetic regions of the M-MuLV genome: *gag* (the internal virion proteins), *pol* (the reverse transcriptase), and *env* (the glycoprotein). The nonproducer cell line isolated in these studies is infected with a deleted M-MuLV genome that makes no normal *gag* or *pol* products but has an apparently normal *env* gene product and a normal *env* mRNA.

MATERIALS AND METHODS

Cells and leukemia viruses. Most of the cells and viruses have been described previously. JLS-V11 cells (58) were provided by Electronucleonics Inc., Bethesda, Md., 18 passages after their infection with M-MuLV. JLS-V9 (V9) cells (58) were obtained from Kenneth Manly (Roswell Park Memorial Institute). NIH/3T3 cells (26) were a gift of S. A. Aaronson (National Institutes of Health). BALB/3T3 cells clone A31 (1) were obtained from R. Pollack, and the 8c subclone 81 cat cells, a sarcoma-positive leukemia-negative (S+L−) cat cell line transformed by Moloney murine sarcoma virus (17), was kindly provided by P. Fischinger. NIH/3T3 cells producing M-MuLV clone 1 virus were derived in this laboratory (16). XC cells (52) were provided by J. Hartley (National Institutes of Health). Cells were grown in Dulbecco-modified Eagle medium with 10% calf serum (NIH/3T3, BALB/3T3, XC, and NIH cells producing M-MuLV) or with 10% heat-inactivated fetal calf serum (JLS-V9, JLS-V11, and all subclones derived from JLS-V11). The cat S+L− cells were grown in McCoy SA medium plus 14% heat-inactivated fetal calf serum.

M-MuLV infections and direct plaque assay. Plaque assays were performed with the XC cell fusion procedure (44). For quantitation of $MuLV_X$ (xenotropic MuLV), the focus assay described by Fischinger et al. (17) was used. Reverse transcriptase assays of

supernatants were done as described elsewhere (20).

Infective center assays were carried out by a modification of a published procedure (26). Sensitive BALB/3T3 cells were plated at 10^5 cells per 6-cm dish. The next day, cells to be tested were trypsinized for 10 min with 0.5% trypsin at room temperature, diluted into medium, and then plated onto the indicator cells at various densities. After 3 days the cultures were UV irradiated and overlaid with XC cells. The plating efficiency of the cells was determined separately. JLS-V9 and JLS-V11 routinely gave plating efficiencies of 60 to 80%.

Antisera. Fluorescein-conjugated antibody to Tween-ether-disrupted M-MuLV made in goats and rhodamine counter stain were obtained through the Virus Cancer Program. Anti-Friend MuLV gp70 serum made in rabbits was kindly provided by D. Bolognesi. Goat anti-rabbit immunoglobulin and normal rabbit serum were purchased from Meloy Laboratories. Anti-MuLV p30 serum made in rabbits was kindly provided by David Livingston. Anti-M-MuLV p30 and gp69/70 antisera made in rabbits were prepared in this laboratory (39).

Radioimmunoassays. The cell extracts were prepared as follows. Subconfluent plates of cells (15 cm) were harvested with phosphate-buffered saline (PBS) (pH 7.4)-2 mM EDTA and then washed three times with PBS. Cells were stored as 20% cell packs at −70°C. The cell packs were disrupted in 1% Triton X-100 and 0.5% sodium deoxycholate, and the nuclei were collected by centrifugation at 2,000 rpm for 4 min. The supernatant was then extracted twice with ether, and the aqueous phase was dialyzed with 0.05 M Tris-hydrochloride (pH 7.8) and used for the radioimmunoassay. The radioimmunoprecipitation assays for the p30 protein were performed as described by Parks et al. (38). Protein determinations were done by the Lowry (31) method with bovine serum albumin as a standard. The first incubation was carried out in 450 μl of 0.1 M $NaPO_4$, (pH 7.5), 1% normal rabbit serum, 0.01% Triton X-100, 0.01 M EDTA, competing antigen and rabbit anti-M-MuLV p30 for 1 h at 37°C, and then 50 μl of ^{125}I-p30 (10,000 cpm) in 0.1 M $NaPO_4$ (pH 7.5), 0.01% Triton X-100, 0.01 M EDTA, and 1% normal rabbit serum was added and incubated for another hour at 37°C. Finally, 30 μl of goat anti-rabbit immunoglobin serum was added, and the mixture was incubated for 1 h at 37°C. The precipitate was collected by centrifugation for 10 min at 3,000 cpm. The supernatant was decanted, and the pellet was washed with 0.5 ml of 1-mg/ml bovine serum albumin-0.1% Nonidet P-40 in PBS. The precipitate was again collected by centrifugation. This washing procedure was repeated three times. The tubes were then counted in a gamma-ray counter.

Immunofluorescence. Cytoplasmic staining was carried out as described previously (20).

Membrane staining of live cells. The procedures for staining antigens on the surface of live cells were provided by Nancy Hopkins (unpublished data). Cover slips with cells were washed five times in PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS-MC). A 25-μl amount of a 1/40 dilution of fluorescein-conjugated anti-M-MuLV antibody and a 1/100 dilution of rhodamine counterstain in PBS-MC was added, and the cover slips were incubated for 30 min in a moist chamber at 37°C. They were then washed five times in PBS-MC and mounted onto slides with PBS-MC and 50% glycerol.

For membrane staining of formaldehyde-fixed cells, cover slips with cells were washed five times in PBS and then fixed for 20 min in PBS containing 3.5% formaldehyde at room temperature. The cover slips were then washed five times in PBS and stained as described in the procedure for acetone-fixed cells.

The method used for indirect staining with nonconjugated rabbit anti-glycoprotein antiserum was as follows. For membrane staining of live cells, cover slips with cells were washed with PBS-MC, 25 μl of a 1/20 dilution of anti-glycoprotein rabbit antiserum in PBS-MC were added, and incubation was carried out for 30 min at 37°C in a moist chamber. The cover slips were then washed five times in PBS-MC, 25 μl of a 1/40 dilution of fluorescein-conjugated goat anti-rabbit antiserum and a 1/100 dilution of rhodamine counterstain in PBS-MC was added, and the second incubation was carried out for 30 min at 37°C. The cover slips were washed extensively and mounted as described above. For cytoplasmic staining of acetone-fixed cells and for membrane staining of formaldehyde-fixed cells, the procedure was modified accordingly.

Metabolic labeling and gel electrophoresis. Cultures about 70% confluent (10-cm plates) were labeled for 2 h with [^{35}S]methionine (New England Nuclear Corp., 50 μCi/ml) in Dulbecco-modified Eagle medium containing 1/50 the normal methionine concentration and 10% dialyzed heat-inactivated fetal calf serum. The cells were lysed with 0.01 M phosphate (pH 7.2), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 0.15 M NaCl. The lysates were scraped from the plates and clarified by centrifugation for 10 min at 1,000 × *g*.

To reduce nonspecific background in the immunoprecipitation, the lysates were precleared by the technique of Witte et al. (56). Extracts were incubated overnight with 10 μl of normal rabbit serum at 4°C and then for 1 h at 4°C with 100 μl of 10% (wt/vol) Formalin-fixed *Staphylococcus aureus* as previously described (28). The components bound to *S. aureus* were collected by centrifugation for 30 min at 35,000 rpm in a Beckman ultracentrifuge.

The supernatants were then immunoprecipitated by overnight incubation at 4°C with the appropriate antiserum followed by 1 h of incubation with *S. aureus* (as above). The precipitates were washed three times with lysis buffer, resuspended in 50 μl of 50 mM Tris (pH 6.8)-1% 2-mercaptoethanol-1% SDS-10% glycerol-bromophenol blue, boiled for 5 min, and analyzed on 5 to 20% gradient slab gels (29). The gels were stained and dried, and the labeled bands were visualized by fluorography and autoradiography at −70°C (9).

Extraction and analysis of cytoplasmic RNA. Cytoplasmic RNA from producer and nonproducer cells was prepared as described previously (14) and by the urea-SDS method (19) as described by Sharp et al. (46). Polyadenylic acid-containing RNA was prepared by affinity chromatography on oligodeoxythymidylic acid-cellulose (T3, Collaborative Research)

(3). Cytoplasmic RNAs were analyzed in 15 to 30% sucrose gradients containing 0.1% SDS as described previously (14). Purified polyribosomes were prepared as described previously by sedimenting polyribosomes from cytoplasmic extracts through 1 and 2 M sucrose (15, 18). Electrophoresis of RNA on denaturing methylmercuric hydroxide agarose gels was performed as described by Bailey and Davidson (4) with a slab gel apparatus (20 by 12 by 0.3 cm). Methylmercuric hydroxide was obtained from Alfa Products (Danvers, Mass.) as a 1 M stock solution.

Preparation of M-MuLV cDNA and hybridization techniques. ^{3}H-labeled M-MuLV complementary (cDNA) was prepared by incubation of M-MuLV clone 1 virions (16) in vitro in the endogenous RNA-dependent DNA polymerase reaction, as described previously (13). The specific activity of the M-MuLV cDNA was 1.6×10^7 cpm/μg as calculated from the specific activity of the radioactive deoxyribonucleoside triphosphate precursor. ^{3}H-labeled M-MuLV cDNA that had been depleted of uninfected BALB/c cell complementary sequences was a kind gift of Rudolf Jaenisch, and its preparation is described by Jaenisch (23).

^{32}P-labeled M-MuLV cDNA was prepared from M-MuLV grown in NRK cells (CP1 cells, E. Rothenberg, unpublished data) according to Taylor et al. (53) in the presence of calf thymus oligodeoxynucleotide primers. The initial specific activity of the cDNA was 10^8 to 2×10^8 cpm/μg as calculated from the specific activity of the labeled precursor.

Hybridization of cytoplasmic RNAs with M-MuLV cDNA to measure extent and concentration of virus-specific RNA sequences was as previously described (13). Briefly, the RNA samples were combined with ^{3}H-labeled M-MuLV cDNA (500 to 1,000 cpm) and incubated for the times indicated. Annealing conditions were 0.3 M NETES (0.3 M NaCl, 0.01 M TES, pH 7.5, 1 mM EDTA)-0.1% SDS at 66°C, and the total reaction volume was 5 to 7 μl. After annealing, the samples were expelled into 50 μl of S1 nuclease reagent (40 U of S1 nuclease per ml, 0.25 M K acetate [pH 4.5], 20 μg of denatured calf thymus DNA per ml, 0.01 M $ZnSO_4$), and the unhybridized cDNA was digested for 30 to 60 min at 45°C. The S1-resistant material was precipitated with trichloroacetic acid, and radioactivity in precipitated material was measured by filtration (Millipore Corp.) and liquid scintillation counting.

Hybridization across sucrose gradients was performed as described (13). Briefly, samples of sucrose gradient fractions were adjusted to annealing conditions and M-MuLV cDNA (500 to 1,000 cpm) was added to each sample. After incubation of all of the samples for the same length of time, the hybridized cDNA was measured and converted to relative virus-specific RNA concentration as described before (13).

Transfer of RNA from agarose gels to diazobenzyloxymethyl paper. The aminobenzyloxymethyl paper was prepared from 1-[(*m*-nitrobenzyloxy)methyl]pyridium chloride and stored in a desiccator at 5°C. Immediately before the transfer was started the paper was diazotized at 4°C. The methylmercuric hydroxide agarose gel was quenched with 0.5 M NH_4Ac and stained with 10 μg of ethidium bromide per ml. The gel was then placed into 200 ml of 50 mM NaOH-5 mM 2-mercaptoethanol and rocked gently for 30 min at room temperature. The gel was then treated with 200 ml of 200 mM potassium phosphate (pH 6.5) containing 7 mM iodoacetic acid for 10 min at room temperature and then twice with 200 ml of 25 mM potassium phosphate (pH 6.5) for 5 min at room temperature. After treatment as above the procedure for transfer was essentially the same as the one described by Southern (48) except that 25 mM potassium phosphate (pH 6.5) was used. Transfer was allowed to proceed overnight. After the transfer the paper was washed twice in 200 ml of water, dried in air, and stored at 5°C until used for hybridization. For hybridization to the RNA bound to the paper the procedure was the same as described by Alwine et al. (2). The paper was pretreated overnight at 42°C with hybridization buffer (50% formamide, 0.75 M sodium chloride, 75 mM sodium citrate) containing 0.02% (wt/vol) each of bovine serum albumin, Ficoll, and polyvinylpyrolidone; 1.5 mg of denatured calf thymus DNA per ml, and 1% (wt/vol) glycine. Hybridization was then performed in the same mixture without the glycine and the probe (200,000 cpm/2 ml) for 36 h at 42°C. The paper was then washed with six to eight changes of hybridization buffer at 37°C each for 30 min with rocking and then with three changes of 50 mM sodium chloride-3 mM sodium citrate at 4°C. The paper was blotted to remove excess solution, wrapped in Saran Wrap, and visualized by autoradiography.

RESULTS

JLS-V9 is a cell line that was derived from BALB/c bone marrow; on infection with M-MuLV these cells were designated JLS-V11 (58). JLS-V11 cells were received in our laboratory at the 18th transfer, and by the 40th transfer virus production had dropped markedly as measured by the amount of reverse transcriptase activity, or particles banding at 1.16 g/cm^3, in the medium. We were interested to determine whether this alteration in virus production was due to a general decrease in virus production in all cells, or whether it was the result of selection of virus-negative cells present in the original JLS-V11 culture. An infective center assay at passage 121 revealed that only 0.4% of the cells were producing M-MuLV, but the virus-positive cells produced normal quantities of virus. Therefore, it appeared that we were dealing initially with a heterogenous population of cells, and thus we cloned early and late passage JLS-V11 cells.

In a clone isolated from passage 21 of JLS-V11 cells (hereafter referred to as "V11" cells), all of the cells were producing virus as measured by an infective center assay. After culture for more than 1 year (more than 50 transfers), they remained stable producers. From the late transfer JLS-V11 cells (transfer 121), 15 clones were isolated, all of which were negative for the production of type C virus both by release of plaque-

forming virus measured by XC cell assay and by particle-associated DNA polymerase detectable in supernatants. The cells themselves also did not induce syncytium formation when overlaid with XC cells. When stained with fluorescent serum made against disrupted M-MuLV, all the clones stained strongly positive in the cytoplasm as well as on the cell membrane; the uninfected JLS-V9 cells displayed no detectable fluorescence. It appeared therefore that the nonproducer JLS-V11 cells (hereafter referred to as "V11-NP") expressed viral antigens but were not able to produce any virus particles. The purpose of the following experiments was to define the lesion in these cells.

Proteins. The genome of retroviruses codes for three major polyprotein gene products: (i) $Pr65^{gag}$, that is processed to give rise to most of the structural proteins of the virus (5, 45); (ii) $Pr180^{gag\text{-}pol}$, the precursor of p85 (reverse transcriptase) (25, 27, 36); and (iii) $Pr80^{env}$, the precursor of the envelope proteins, mainly the glycoprotein gp70 (12, 34, 57). In characterizing V11-NP cells, we first determined whether any of the viral proteins were expressed.

The quantity of p30, the major structural virion protein, and a cleavage product of $Pr65^{gag}$ was measured by radioimmunoassay (Fig. 1). V11-NP cells had no more p30 antigenic material than uninfected V9 cells (~10 ng/mg), whereas V11 cells producing M-MuLV and V9 cells induced with bromodeoxyuridine to produce endogenous MuLV (8) had high levels of p30 antigen (500 to 1,500 ng/mg). In this radioimmunoassay, iodinated Rauscher MuLV p30 and homologous antibody made against Rauscher p30 protein were used.

To further investigate viral protein synthesis, polyacrylamide gel electrophoresis of specific immunoprecipitates was used (Fig. 2). Extracts of [^{35}S]methionine-labeled V11 cells (slots a to f, Fig. 2), V11-NP cells (slots g to l), and V9 cells (slots m to p) were analyzed with a variety of antisera. V9 cells had no specific proteins. V11 cells showed the array of proteins previously characterized in other systems: $Pr180^{gag\text{-}pol}$ precipitated with anti-reverse transcriptase (slot a) and anti-p30 (slot c) and products from about p145 to p85 precipitated only with anti-reverse transcriptase; $Pr65^{gag}$, p30, and some intermediates precipitated with anti-p30 and anti-whole virus serum (slot e); $Pr80^{env}$ precipitated with anti-gp70 (slot b) and anti-whole virus serum. Some $Pr65^{gag}$ precipitated with the anti-reverse transcriptase serum because a known contaminating activity in the serum reacts with the p15 component of $Pr65^{gag}$ (56). Normal rabbit serum precipitated no proteins (slot f), and p30 competed out all precipitation with anti-p30 (slot d).

V11-NP cells had no detectable $Pr180^{gag\text{-}pol}$ or $Pr65^{gag}$ with the same sera (slots g and i) but did have $Pr80^{env}$ (slot h). The anti-whole virus serum (slot h) precipitated a diffuse band at 70,000 molecular weight that is presumed to be the cleaved form of glycoprotein, gp70. Why the anti-gp70 detected so little of this polypeptide is not known, but this antiserum has previously been shown to be much more avid for $Pr80^{env}$ than for gp70 (47).

A minor component in V11-NP cells, P45, was precipitated with anti-p30 (slot i) and anti-whole virus serum (slot k). The reactivity with anti-p30 was competed out with p30 (slot j). A P45 polypeptide was also evident in V11 cells ex-

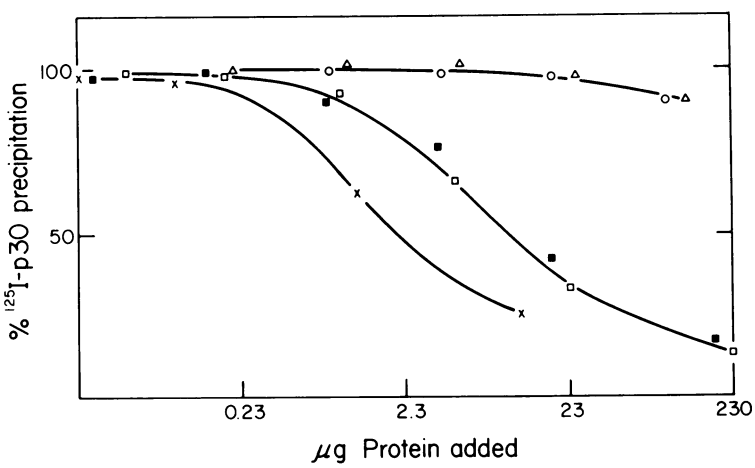

FIG. 1. *p30 radioimmunoassays. Cell extracts from NP (○), JLS-V9 (△), bromodeoxyuridine-induced JLS-V9 (■) and NP (□), and JLS-V11 (×) cells were prepared, and the radioimmunoassay was carried out as described in the text.*

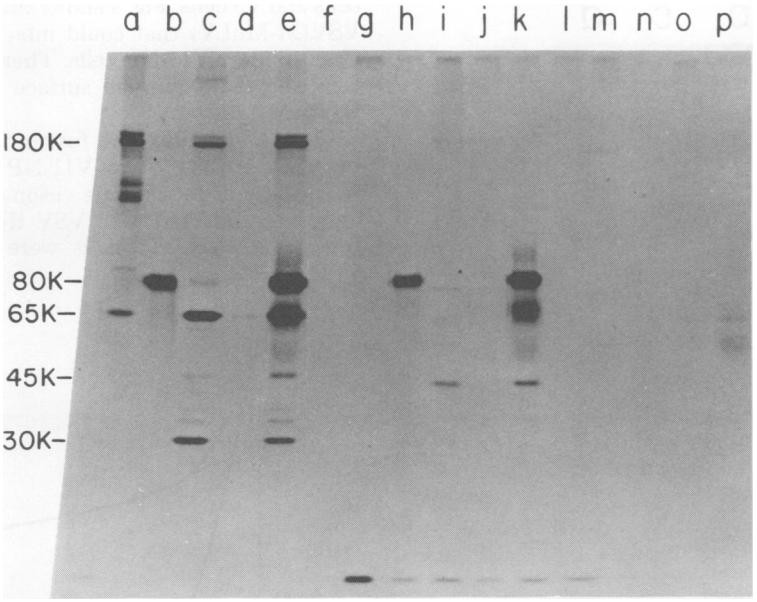

FIG. 2. *Immunoprecipitation of viral proteins from V11 and V11-NP cells. (a to f) Immunoprecipitation of [^{35}S]methionine-labeled V11 cell extracts with (a) anti-reverse transcriptase serum, (b) anti-glycoprotein serum, (c) anti-p30 serum, (d) anti-p30 serum in the presence of an excess of purified, p30 protein, (e) rat anti-whole virus serum, and (f) normal rabbit serum. (g to l) Immunoprecipitation of [^{35}S]methionine-labeled V11-NP cell extracts with (g) anti-reverse transcriptase serum, (h) anti-glycoprotein serum, (i) anti-p30 serum, (j) anti-p30 serum in the presence of an excess of purified, p30 protein, (k) rat anti-whole virus serum, and (l) normal rabbit serum. (m to p) Immunoprecipitation of [^{35}S]methionine-labeled JLS-V9 cell extracts with (m) anti-reverse transcriptase serum, (n) anti-glycoprotein serum, (o) anti-p30 serum, and (p) rat anti-whole virus serum.*

tracts (slots c and e). Its concentration was very much less than that of the other p30-related polypeptides in V11 cells, and we are uncertain whether it derives from the M-MuLV genome or from an endogenous or contaminating virus.

From the immunoprecipitation data, V11-NP cells appear to contain the glycoprotein precursor as well as finished gp70. Immunofluorescent staining of live and acetone-fixed cells with monospecific anti-gp70 antiserum showed that the glycoprotein was found both in the cytoplasm of fixed cells and on the cell membrane of live cells (unpublished data).

To investigate whether both gp70 and Pr80^{env} were localized on the outer cell membrane, V11-NP cells were radioiodinated with lactoperoxidase as catalyst. Immunoprecipitation and polyacrylamide gel electrophoresis showed that only labeled gp70 was precipitated with anti-gp70 (Fig. 3, lane a) even though the antiserum used was more avid for Pr80^{env} than for gp70; neither anti-p30 nor normal rabbit serum precipitated any protein bands (lanes b and c). The size of Pr80^{env} is evident from Fig. 3, lane d. When the labeled cells were trypsinized, 70 to 80% of labeled gp70 was removed, indicating that most of it is accessible to trypsinization (data not shown). V11-NP cells appeared to produce both gp70 and its precursor, Pr80^{env}; only the gp70, however, was found on the outer cellular membrane.

Surface restriction. The virus-negative V11-NP cells were isolated from a virus-producing culture, suggesting that the cells would be resistant to superinfecting MuLV. Otherwise, V11-NP cells should have been superinfected with M-MuLV from producer cells in the original JLS-V11 cultures. To clarify this point, the interference properties of the V11-NP cells were investigated. V11-NP cells and control V9 cells were infected with M-MuLV at a multiplicity of infection of 1, and supernatants were collected 2 and 5 days after infection. V11-NP cells produced about 1,000-fold less virus than V9 cells (Table 1). To examine whether this strong resistance to superinfection occurred at the cell surface, the pseudotype virus, vesicular stomatitis virus (VSV)(M-MuLV), was produced by infecting M-MuLV-producing cells with heat-labile VSV tl-17 (7, 59). The stock was then heated at 45°C for various times to kill VSV(VSV), and residual pseudotypes were titered on V11-NP

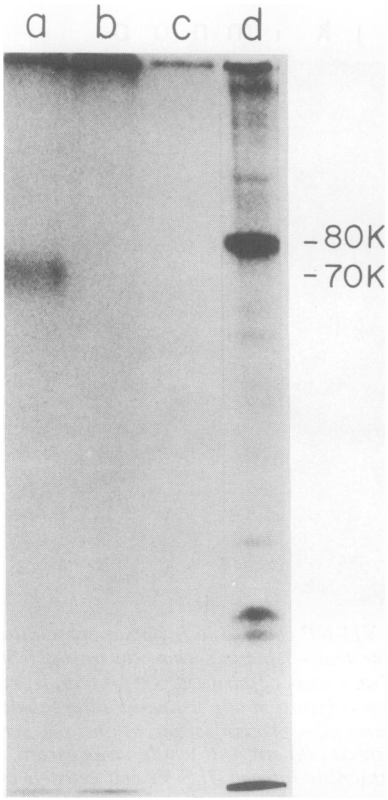

FIG. 3. *Surface iodination of gp70 on V11-NP cells. Surface iodination of the V11-NP cells was essentially carried out as described by Hynes (22). After the iodination was stopped, the cells were washed three times with PBS. The cell pellets were lysed with 0.5 ml of 0.1 M Tris (pH 6.8)–2 mM phenylmethylsulfonylfluoride–15% glycerol–2% SDS. Immunoprecipitation of the cell extracts was carried out with (a) anti-glycoprotein serum, (b) anti-p30 serum, (c) normal rabbit serum; (d) immunoprecipitation of a [^{35}S]methionine-labeled V11 cell extract was carried out with anti-glycoprotein serum.*

TABLE 1. *Interference of NP cells with M-MuLV*[a]

Cell type	Yield of XC cell (PFU/ml per 10^6 test cells) at:	
	2 days after infection	5 days after infection
JLS-V9	8.6×10^5	1.5×10^5
V11-NP	1.3×10^3	1.3×10^2

[a] Cells (5×10^5) were plated onto 5-cm dishes, and on the next day the cultures were infected with M-MuLV at a multiplicity of infection of 1 in the presence of 8 μg of polybrene per ml. After 2 h of adsorption, the inoculum was removed, and the cells were washed two times with regular medium. After 2 and 5 days, 24-h harvests were collected and the virus yield was determined in an XC assay with NIH/3T3 cells as indicators.

cells and V9 cells. Fig. 4 shows that only $10^{-2.5}$ of VSV(M-MuLV) that could infect V9 cells was able to infect V11-NP cells. Therefore, V11-NP cells showed a marked surface restriction towards M-MuLV.

Lack of pseudotype formation. To examine whether the gp70 on V11-NP cells is able to participate in pseudotype virion formation, the cells were infected with VSV tl-17. M-MuLV-producing NIH/3T3 cells were superinfected

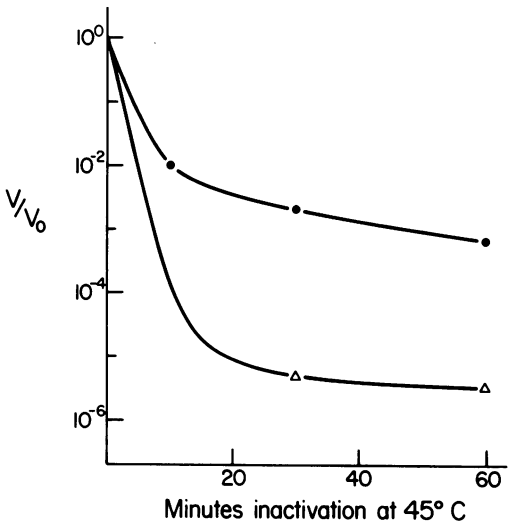

FIG. 4. *Interference of NP cells with VSV(M-MuLV). Pseudotypes of the VSV mutant tl-17 with the coat of M-MuLV were prepared as previously described (7) and inactivated at 45°C for the indicated times. VSV plaque assays (49) were performed with JLS-V9 (●) and NP (△) cells as indicator cells. V/V_0 is the surviving fraction after heating.*

TABLE 2. *Lack of pseudotype formation between VSV and M-MuLV in V11-NP cells*[a]

Cells for heated VSV tl-17 stocks	Surviving VSV fraction on indicator cells:	
	JLS-V9	V11-NP
M-MuLV-infected NIH/3T3	10^{-3}	10^{-6}
V11-NP	3.5×10^{-6}	1.5×10^{-6}

[a] Subconfluent plates of NIH/3T3 cells producing M-MuLV and V11-NP cells were infected with VSV tl-17 at 32°C at a multiplicity of infection of 1 to 2. The virus was absorbed in a volume of 0.5 ml/5-cm dish. After 45 min of adsorption, the inoculum was removed, the cells were washed once with regular medium, and then 5 ml of regular medium was added. After 12 h of infection at 32°C, the virus yield was harvested, and portions were heat inactivated at 45°C for 60 min. The residual fraction of VSV was then determined in a VSV plaque assay on JLS-V9 and V11-NP cells at 32°C.

with VSV tl-17 in parallel, and the two VSV stocks were then heated at 45°C to eliminate the pure VSV in the populations. To detect pseudotypes, the stocks were assayed on V9 cells or V11-NP cells (Table 2). Although the stock grown on the M-MuLV-infected cells had 10^3 PFU of pseudotype VSV per ml that appeared on V9 cells but not V11-NP cells, no such pseudotypes were evident in the VSV preparation made on V11-NP cells. The gp70 on the surface of V11-NP cells thus appears incapable of participating in pseudotype virion synthesis.

Expression of M-MuLV-specific RNA in producer and nonproducer cells. The M-MuLV-specific RNA in V11-NP cells was examined to determine whether the lack of viral proteins in the nonproducer cells was due to an alteration in the expression of virus-specific RNA. Cytoplasmic RNA extracted from producer and nonproducer cells was annealed with M-MuLV cDNA in conditions of virus-specific RNA excess (13). At saturation, RNA from the V11-NP cells could anneal M-MuLV cDNA to the same (maximal) extent as RNA from the producer cells (Fig. 5), indicating that essentially all virus-specific RNA sequences that could be recognized by the M-MuLV cDNA probe were present in the nonproducer cells. In annealing experiments performed in this manner, the virus-specific RNA concentration is inversely proportional to the product of RNA concentration and annealing time that gives half-maximal annealing (the $C_rt_{1/2}$ value) (13). Comparison with the $C_rt_{1/2}$ value for pure M-MuLV 70S RNA (4.02×10^{-2} mol-s/liter at standard annealing conditions [15]) indicated that 0.03% of the cytoplasmic RNA from V11-NP cells and 0.10% of the producer cell cytoplasmic RNA was virus specific. The lack of virus production in the nonproducer cells, therefore, did not result from marked alteration in the expression of virus-specific RNA because no change in extent of M-MuLV-specific sequences present was detected and the concentration of these sequences was only threefold lower than for producer cells.

Uninfected BALB/c mouse cells contain RNA sequences that can anneal with M-MuLV cDNA to a limited extent (6, 8, 14, 15); at least some of these sequences may represent endogenous C-type viruses. To ensure that the annealings of Fig. 5 were analyzing transcription of M-MuLV cDNA and not uninfected cell sequences, producer and nonproducer cell RNAs were annealed with M-MuLV cDNA that had been depleted of sequences that anneal with uninfected BALB/c cell DNA (a kind gift of Rudolf Jaenisch). V11-NP and V11 cell RNA annealed to equal extents to the selected cDNA probe (Fig. 6). The $C_rt_{1/2}$ values indicated that with these RNA preparations, the nonproducer cell cytoplasmic RNA contained approximately one-fourth as much virus-specific RNA as producer cells (0.06 versus 0.26%).

Intracellular virus-specific RNA in cells producing MuLV consists of 38S and 21S virus-specific RNA (11, 13, 33, 43, 54). To examine whether the same virus-specific RNA species were present in the nonproducer cells, cytoplas-

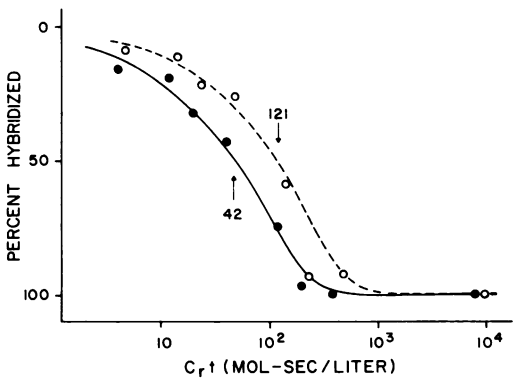

FIG. 5. *Annealing of cytoplasmic RNAs with M-MuLV-specific cDNA. Cytoplasmic RNA extracted from producer and non-producer JLS-V11 cells was annealed with ^{3}H-labeled M-MuLV-specific cDNA for different lengths of time. The percent maximal cDNA hybridized is plotted as a function of the C_rt value (product of RNA concentration and time of annealing) corrected to standard conditions (10). The $C_rt_{1/2}$ values are also indicated. Symbols: ●, producer JLS-V11 cell RNA; ○, nonproducer cell RNA.*

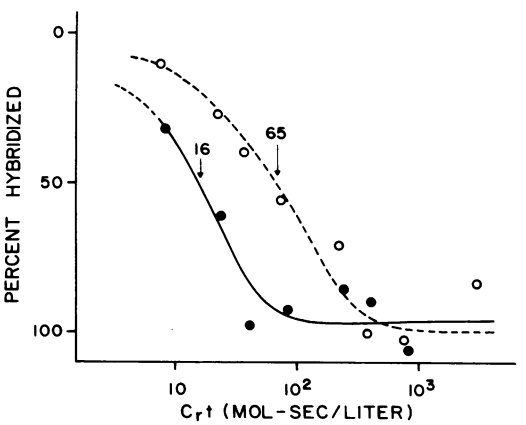

FIG. 6. *Annealing of cytoplasmic RNAs with selected M-MuLV-specific cDNA. Cytoplasmic RNA from producer and nonproducer JLS-V11 cells (different preparations from Fig. 5) was annealed with M-MuLV cDNA which had been depleted of BALB/c uninfected cell sequences. Analysis was the same as in Fig. 5. Symbols: ●, producer JLS-V11 cell RNA; ○, nonproducer RNA.*

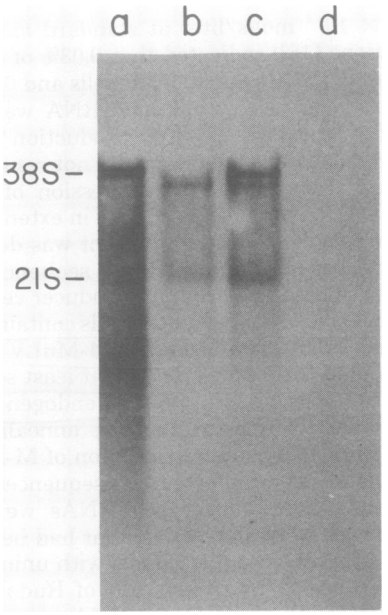

FIG. 7. *Electrophoretic analyses of cytoplasmic RNA from V11-NP cells. A 5-μg amount each of polyadenylic acid-selected cytoplasmic RNA from V11-NP cells, V11 producer cells, and uninfected JLS-V9 cells was electrophoretically separated on a 1% agarose methylmercuric hydroxide gel for 2.5 h at 6 V/cm. The RNA in the gel was then transferred to diazobenzyloxymethyl paper, and the M-MuLV specific bands were visualized by hybridization with ^{32}P-labeled M-MuLV cDNA. The exposure time for the autoradiography was 8 h. (a) Pure M-MuLV RNA; (b) V11-NP RNA; (c) V11 producer RNA; (d) JLS-V9 RNA.*

mic RNA extracted from V11-NP was separated by electrophoresis through denaturing methylmercuric hydroxide agarose gels. The RNA was transferred from the agarose gel to diazobenzyloxymethyl paper, an affinity paper for nucleic acids described by Alwine et al. (2). The virus-specific RNAs covalently linked to the paper were then detected by hybridization with ^{32}P-labeled M-MuLV cDNA. The largest viral RNA in the V11-NP cells (Fig. 7, lane b) was 1,000 nucleotides shorter than that of wild-type M-MuLV RNA (Fig. 7, lane a); the subgenomic 21S RNA was present in both the M-MuLV-producing cells (Fig. 7, lane c) and the V11-NP and was apparently the same size in the two cell lines. V11 producer cells contained the regular genomic 38S RNA, subgenomic 21S RNA, and an RNA that comigrated with the larger virus-specific RNA of the V11-NP cells (Fig. 7, lane c). Uninfected cells showed no RNA bands by this procedure (Fig. 7, lane d).

The V11-NP cells therefore appear to contain a genome having a 1,000-nucleotide deletion. The deletion, though, does not affect the size of the 21S subgenomic RNA, implying that the deletion occurs outside of the regions covered by this RNA. The inability to detect the deletion by liquid hybridization experiments (Fig. 5 and 6) is probably a consequence of its representing only 11% of the 9,000-nucleotide genome. The V11 producer cells contained both full-length 38S RNA as well as a species with most likely the same deletion as the one found in the V11-NP cells. Virus produced by the V11 cells also contains full-length 38S RNA and the deleted molecule (P. Besmer, unpublished data).

To determine whether the deleted genomic RNA as well as the 21S subgenomic RNA was contained in polyribosomes, the polyribosome fraction of the V11 and V11-NP cells was treated with or without EDTA and analyzed by sedimentation through sucrose gradients. Virus-specific RNA in the "released messenger region" of the sucrose gradients (50 to 200S) was further analyzed by sedimentation through sucrose-SDS gradients, and virus-specific RNA appearing in this region after EDTA disruption of polyribosomes was scored as functional mRNA. (15). By this criterion polyribosomes from the V11-NP and the V11 cells contained both genome size 35 to 38S and subgenomic 21S mRNA's (Fig. 8). The deleted genome RNA from the V11-NP cells is therefore found on polyribosomes as well as the subgenomic 21S RNA.

Can the virus genome in the V11-NP cells be rescued? Previously we have shown that uninfected V9 cells can be induced to produce the BALB/c endogenous xenotropic ($MuLV_X$) and ecotropic ($MuLV_E$) virus upon treatment with halogenated pyrimidines (8). The N-tropic MuLV does not form XC cell plaques when assayed in an XC assay with NIH/3T3 cells as indicators (50). V11-NP cells were treated with bromodeoxyuridine to determine whether the compound could induce the endogenous V9 viruses, which might then rescue or complement the M-MuLV contained in the V11-NP cells. The virus yield 2 days after removal of the halogenated pyrimidine was determined (Table 3). V9 cells upon induction produced 3×10^5 focus-forming units of $MuLV_X$ per ml (as measured by a focus assay on S+L− cat cells) and no XC cell plaques on NIH cells. The V11-NP cells upon induction produced 3×10^5 focus-forming units of $MuLV_X$ per ml but also made 50 to 100 PFU of virus per ml that gave XC cell plaques on NIH cells. In an attempt to separate this low amount of XC plaque-forming virus from the

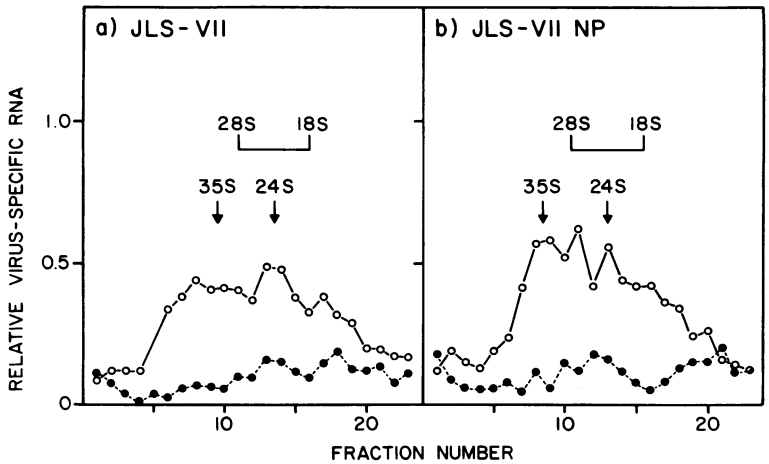

FIG. 8. *Size of M-MuLV-specific mRNA in JLS-V11 and V11-NP cells. (a) JLS-V11 cells. Purified polyribosomes were prepared from 10 15-cm tissue culture dishes of JLS-V11 cells at half confluency. Before extraction the cells had been treated with 1 μg of cycloheximide per ml for 30 min to increase the size of the polyribosomes. The polyribosomes were divided in half, and one half was treated with 10 mM EDTA to disaggregate them. The two samples were then sedimented in a sucrose gradient to display the polyribosomes (not shown), and fractions containing the "released messenger" regions (50 to 200S) were pooled. RNA was extracted from the two pooled samples and sedimented in a 10.5-ml 15 to 30% sucrose gradient in SDS buffer containing 0.1% SDS. After centrifugation in a Beckman SW41 rotor for 12 h at 24,000 rpm and 25°C, the gradients were fractionated, and 100-μl portions of each fraction were concentrated by ethanol precipitation into 50 μl of 0.3 M NETES-0.1% SDS buffer. ^{3}H-labeled M-MuLV cDNA (550 cpm) was added to each concentrated fraction and annealing was performed at 66°C for 18 h. After annealing, the samples were digested with S1 nuclease, and the amount of S1-resistant radioactivity was determined. The relative amount of virus-specific RNA present in each fraction was calculated from the percentage of cDNA hybridized as before (a relative virus-specific RNA concentration of 1 corresponds to 50% cDNA probe annealed) (13). The data from the two sucrose gradients are superimposed, and the locations of the 28S and 18S rRNA's (determined by monitoring the sucrose gradient for absorbance at 260 nm during fractionation) are also indicated. Symbols: ○, data from the EDTA treated polyribosomes; ●, data from the control polyribosomes. (b) V11-NP cells. A similar experiment was performed with 10 15-cm dishes of V11-NP cells at half-confluency.*

TABLE 3. *Rescue of M-MuLV from V11-NP cells upon induction with bromodeoxyuridine*[a]

Cells	XC plaques/ml	$MuLV_X$ (FFU/ml)
JLS-V9	0	3×10^5
V11-NP	50–100	3×10^5

[a] Bromodeoxyuridine induction of V11-NP cells was performed as described previously (8). Two days after the removal of the bromodeoxyuridine-containing medium from the cultures, a 24-h harvest was collected, and the XC plaque titer on NIH/3T3 indicator cells and $MuLV_X$ titer on cat S+L− cells were determined. FFU, Focus-forming units.

TABLE 4. *Characterization of virus isolated from the bromodeoxyuridine-induced virus of V11-NP cells*[a]

Isolate	Virus titer on:	
	NIH cells (XC plaques/ml)	BALB/c 3T3 cells (XC plaques/ml)
1	2.6×10^5	6×10^3
2	4.3×10^5	6×10^3

[a] NIH/3T3 cells were plated at 5×10^5 cells per 5-cm dish, and on the next day the cultures were infected with bromodeoxyuridine-induced virus from V11-NP cells in the presence of 8 μg of polybrene per ml. At 5 days after infection, a 24-h harvest was used to infect a new culture of NIH/3T3 cells, and this procedure was repeated three times. From the third infected culture, 5 days after the initial infection a 24-h harvest was collected. The respective XC plaque titers of these isolates were then determined on NIH/3T3 cells and on BALB/c 3T3 cells.

endogenous non-plaque-forming N-tropic virus, 5×10^5 NIH cells were infected with the induced virus from NP cells. At 5 days after the infection, the supernatant was used to infect a new culture of NIH cells, and this procedure was repeated three times. A virus stock was then prepared and titered on NIH and BALB/c cells. Two independent virus isolates derived were N-tropic and XC plaque-forming viruses and not NB-tropic as would be expected for M-MuLV (Table 4). These N-tropic viruses thus have the host range marker of the endogenous BALB/c N-

tropic virus (30, 40) and the large XC plaque morphology marker of M-MuLV (44). They may be recombinants between the endogenous N-tropic MuLV and the defective M-MuLV in V11-NP cells. Recombinants involving the Fv-1 host range marker and the XC plaque morphology marker have been described previously (21).

DISCUSSION

Origin of V11-NP cells. The V11-NP cell line appears to have arisen from infection by a deletion mutant of M-MuLV. This deletion probably existed in the stock M-MuLV used to infect JLS-V9 cells and generate the parental JLS-V11 cell culture. Both the stable producer V11 clone studied here and the V11-NP clone contained this M-MuLV RNA lacking 1,000 nucleotides; the producer also had full-size M-MuLV RNA. If V11-NP had been derived as a mutant from V11, then the deleted RNA would probably not have been found in the producer cells.

The V11 producer cells, once cloned, have been stable producers for more than 100 generations thereafter. Thus, there is no tendency to lose the nondeleted genome, and we conclude that the V11-NP cells probably did not ever have a nondeleted genome. The V11-NP cells appear to have a slight growth advantage such that they could overgrow the producer cells in the original mixed culture, and they are resistant to superinfection by M-MuLV. These two properties most likely were essential for the isolation of the V11-NP cells.

Nature and consequences of the deletion in V11-NP cells. The V11-NP cells have both deleted genome-size RNA (vRNA) and normal-size 21S RNA on polyribosomes. They express *env* normally, making an intracellular Pr80 that cleaves to a surface gp70. Thus, the 21S RNA, known to be the glycoprotein mRNA (54), appears to be unchanged in structure and function by the deletion. By contrast, no $Pr65^{gag}$ or $Pr180^{gag\text{-}pol}$ is evident in the cells, implying that the deletion has interferred with their expression. RNA indistinguishable from vRNA is known to be the mRNA for the *gag-pol* products (27, 33, 54). The P45 protein in the V11-NP cells could be the product of the mRNA function of the deleted vRNA in the polyribosomes. If this protein is the N-terminal fragment of the normal *gag* polypeptide, it should have p15, p12, and some of p30. The deletion may therefore occur within the p30 gene. We are presently analyzing P45 further, but it is worth noting that this fragment is just the size of the *gag* fragment found in the product of the Abelson MuLV genome (56). In both V11-NP and Abelson MuLV-infected cells, the product is immunoprecipitated by anti-p30 antiserum but does not register in radioimmunoassays (37). Thus, the deletion mutant described here either could be the parent of Abelson MuLV or could represent a site at which deletions or substitutions frequently occur. The virus in V11-NP cells might even have a partially substituted genome.

An implication from this analysis is that the splicing event that makes the 21S mRNA can occur even though the intervening region has been partly deleted. This conclusion is subject, however, to the reservation that different integrated viral genomes may give rise to the vRNA and to 21S mRNA in V11-NP cells.

Lack of VSV pseudotype production by V11-NP cells. Although V11-NP cells have gp70 on their surface, numerous attempts to produce VSV psuedotypes in these cells have failed (Table 2). This result supports an earlier conjecture (55) that to have a glycoprotein incorporated into the envelope of a virus requires both the glycoprotein and a submembrane protein. The lack of the submembrane $Pr65^{gag}$ in V11-NP cells presumably prevents gp70 from participating in pseudotype formation.

Mutability of the M-MuLV genome. In previous work (20, 35, 41, 42, 47), we and others have characterized five nonconditional mutant classes of MuLV's that are phenotypically different from the deletion in V11-NP (see reference 47). These other mutants arose from carefully cloned MuLV's in one passage, implying a very high rate of spontaneous mutations during MuLV passage. At least one of the five classes represented a deletion mutant. That deletion (M23) abolished both *pol* and *env* function but left *gag* function intact (47). The ability to derive cell lines with a range of M-MuLV mutants should facilitate analysis of the roles of the various gene products in the life of the virus. The high rate of generation of mutants, however, must be considered in any explanations of the behavior retrovirus-infected cells.

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